We suggest on the basis of this study that due to the bitter flavonoids levels are especially influenced by the parents and should be considered when conducting crosses. This study shows that ploidy level influences flavonoid content.

Literature Cited

Cameron, J. W., R. K. Soost and E. O. Olson. 1964. Chimeral basis for color in pink and red grapefruit. J. Hered. 55:23-28.

Cameron, J. W. and R. K. Soost. 1969. Characters of new populations of *Citrus* polyploids, and the relation between tetraploidy in the pollen parent and hybrid tetraploid progeny. Proc. First Intern. Citrus Symp. 1:199-205.

Cameron, J. W. and R. K. Soost. 1968. Genetics, breeding, and nucellar embryony. In: Reuther, W., L. D. Batchelor and H. J. Webber (Eds.) The Citrus Industry Vol. 1, Univ. Calif., Div. of Agric. Sci., Berkeley. 325-370.

Dakora, F. D. 1995. Plant flavonoids: biological molecules for useful exploitation. Australian J. of Plant Physiol. 22:87-99.

Dixon, R. A., A. D. Choudhary, K. Dalkin, R. Edwards, T. Fahrendorf, G. Gowri, M. J. Harrison, C. J. Lamb G. J. Loake and C. A. Maxwell. 1992. Molecular biology of stress-induced phenylpropanoid and isoflavonoid biosynthesis in alfalfa. In: Phenolic Metabolism in Plants, H. A. Stafford and R. K. Ibrahim, Eds., Plenum Press, New York, N.Y, 91-138.

Dixon, R. A. and N. L. Paiva. 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085-1097.

Ebel, J. 1986. Phytoalexin synthesis: the biochemical analysis of the induction process. Annual Review of Phytopathology 24:235-264.

Ebel, J. and A. Mithofer. 1998. Early events in the elicitation of plant defence. Planta 206:335-348.

Hahlbrock, K. and D. Scheel. 1989. Physiology and molecular biology of phenylpropanoid metabolism. Ann. Rev. of Plant Physiol. and Mol. Biol. 40:347-369.

Heller, W. and G. Forkmann. 1993. Biosynthesis of flavonoids. In: J. B. Harborne (Ed.), The Flavonoids: Advances in Research Since 1986. Chapman & Hall, London, 499-535.

Laks, P. E. and M. S. Pruner. 1989. Flavonoid biocides: structure/activity relations of flavonoid phytoalexin analogues. Phytochemistry 28:87-91.

Nahrstedt, A. 1990. The significance of secondary metabolites for interactions between plants and insects. Planta Medica 55:333-338.

Nicholson, R. L. and R. Hammerschmidt. 1992. Phenolic compounds and their role in disease resistance. Annual Review of Phytopathology 30:369-389.

Snyder, B. A. and R. L. Nicholson. 1990. Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. Science 248; 1637-1639.

Soost, R. K. and J. W. Cameron. 1981. 'Oroblanco', a triploid pummelo-grapefruit hybrid. Proc. Intern. Soc. Citriculture 1:59-60.

Vandercook, C. E., R. L. Price and C. A. Harrington. 1975. Multiple automated analyses for orange juice content: determination of total sugars, reducing sugars, total acidity, total amino acids and phenolics. J. Assoc. Official Analytical Chemists. 58:482-487.

Vandercook, C. E. and B. Tisserat. 1989. Flavonoid changes in developing lemons grown in vivo and in vitro. Phytochemistry 28:799-803.

Proc. Fla. State Hort. Soc. 111:260-263. 1998.

A DISCUSSION OF IN VITRO CONTAMINATION CONTROL OF EXPLANTS FROM GREENHOUSE AND FIELD GROWN TREES

MICHAEL G. BAUSHER AND RANDALL P. NIEDZ
U.S. Department of Agriculture, ARS, USHRL
2120 Camden Road
Orlando, FL 32803

Additional index words. Plant tissue culture, sterilization, biocides, isothiazolones, antimicrobial, hypochlorous acid, stabilized chlorine isocyanurates.

Abstract. **Controlling fungal and bacterial contamination of woody plant material is important and extremely difficult from field sources. Greenhouse grown material has less decontamination problems with the field material. Isothiazolone biocides and sodium dichloroisocyanurate (NaDCC) were used singly and in combination to reduce microbial contamination to less than 5% in bud explants derived from field-grown citrus trees. Coating the explant with a slurry containing 20 mL·L^{-1} Plant Preservative Mixture (PPM), a mixture of two isothiazolones, and culturing on medium containing 5 mL·L^{-1} PPM resulted in 63% clean explants compared to >90% contamination with standard disinfestation procedures. Explants treated for 48 hr with 100 or 300 ppm NaDCC resulted in 83% and 96% clean explants, respectively. Phytoxicity problems were present at the 20 mL·L^{-1}.**

In vitro contamination by fungi, bacteria, or yeast is one of the most serious problems of commercial and research plant tissue culture laboratories (Leifert et al., 1994). The establishment of an in vitro culture requires the removal of culturable fungal and bacterial contaminants. Contamination can be especially troublesome form field obtained materials. The inability to adequately control contamination levels is the primary reason for failure of commercial laboratories (Leifert and Woodward, 1997). Chemical methods used include antibiotics and fungicides, alcohols, mercuric chloride, and oxidizing biocides such as halogen compounds (e.g., chlorine, bromine, and iodine) and hydrogen peroxide. Each method used balances factors such as the plant species, type of explant, phytoxicity, type of contaminant(s), and cost to obtain desired results.

Isothiazolones are a class of industrial biocides that have been used prophylactically in the form of Plant Preservative Mixture (PPM) in tissue culture medium to control microbial contamination (Niedz, 1998). PPM contains a mixture of two isothiazolones, methylchloroisothiazolinone and methylisothiazolinone (Guri and Patel, 1998). Adequate control of microbial contamination was achieved as long as inoculum levels were low. Also, little phytotoxicity was observed even at the highest concentrations tested of 2 mL·L^{-1}, twice the highest recommended rate by the manufacturer (Niedz, 1998). In this study, significantly higher isothiazolone levels are used to control the inoculum types and levels associated with greenhouse and field-grown plant material.

Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable. We thank Ms. Delores Lomberk, Mr. Scott Hyndman, and Mr. Gerald Mozoruk for their assistance in the collection, preparation, and in vitro culture of plant material, and Dr. Assaf Guri of Plant Cell Technology for generously providing PPM samples for testing.

Some of the most common sterilants used in plant tissue culture are chlorine derivatives, generally in the form of inorganic sodium or calcium hypochlorite. Regardless of the form of chlorine used when diluted in water hypochlorous acid (HOCl) the primary sterilant is formed. Sodium dichloroisocyanurate (NaDCC) is an organic form of chlorine with a pH of about 7 in solution. At this pH HOCl ions make up 80% of the ionic constitution. NaDCC has been used to sterilize plant material for tissue culture (Parkinson et al., 1996; Pink and Walkey, 1984) and to control bacterial growth in cut flower vase water (Marousky, 1977). We report preliminary studies of the effective use of NaDCC singly and in combination with PPM in removing culturable contaminants from greenhouse and field grown citrus buds.

Materials and Methods

Plant Material. Three- to four-year-old greenhouse grown sweet orange trees (*Citrus sinensis* L. Osbeck cv. Madame Vinous) and six- to eight-year-old field grown sweet orange trees (*C. sinensis* cv. Valencia) were used as bud explant sources for decontamination experiments. Other experiments used explants from field grown Grapefruit trees (C. paridisi cv. Marsh). All bud sticks from greenhouse or field trees were taken from fully expanded flush of previously pruned trees. Buds were excised as 2-3 cm segments, disinfested, and cultured singly in 60 × 15 mm polystyrene culture dishes containing MS (Murashige and Skoog, 1962) salts and vitamins plus 3% (w/v) sucrose. The medium was adjusted to pH 5.8, 8 $g \cdot L^{-1}$ agar added, and then autoclaved for 20 min at 120 C and 104 kPa. Cultures were maintained at a constant 27°C under cool-white fluorescent lamps (30 $\mu mol \cdot s^{-1} \cdot m^{-2}$) for a 16 hr photoperiod. Each treatment contained at least 10 dishes, and the experiment was repeated one to three times. Contamination data was compiled from 30 days after culture and 15 days incubation in nutrient broth (NB) except when noted. NB was prepared with 17.2 $g \cdot L^{-1}$ nutrient broth (Difco Laboratories, Detroit, MI), 10 $g \cdot L^{-1}$ yeast extract, and 2.5 $g \cdot L^{-1}$ glucose.

PPM Treatments. PPM (Guri and Patel, 1998) was obtained from Plant Cell Technology (Washington, DC) and was added to the medium after autoclaving. PPM was added to MS culture medium containing 0.2% agar or 0.8% agar at a concentration of 0 or 20 $mls \cdot L^{-1}$, or 0, 5, 10, or 20 $mls \cdot L^{-1}$, respectively. A 'standard' disinfestation procedure was developed as a baseline to determine the relative effectiveness of PPM. Explants were first treated with the standard disinfestation procedure as follows: bud nodes were cut and air-dried for 1 hr, a 1 min dip in 1 N sulfuric acid immediately followed by a dip in 1 M sodium bicarbonate, and a second one min dip in 1 M sodium bicarbonate 30 min soak in 20% commercial bleach (5.25% sodium hypochlorite) with 0.1% Silwet L-77 (OSI Industries, Terrytown, NY) as a wetting agent, and 3 rinses with sterile distilled water. All disinfestation agents were added to a 500 ml jar and agitated with gentle stirring except where noted. After the standard disinfestation, explants were dipped into 0.2% agar slurry and placed onto culture medium. The slurry dip treatment included two concentrations of PPM and the medium contained four concentrations of PPM. This resulted in a factorial arrangement of explants on PPM-containing slurry and medium. Data was collected as percent contamination from two experiments with 12 replications each, transformed by arcsine, and analyzed by standard ANOVA.

Incubation of explants in a suspension culture containing PPM was also examined. Explants were prepared as before with the standard disinfestation treatment, but were placed into flasks containing liquid MS medium containing 0, 5, 10, or 20 $mL \cdot L^{-1}$ PPM for five days. Explants were then removed and cultured on medium containing no PPM.

Sodium dichloroisocyanurate treatments. Dichloroisocyanuric acid (NaDCC) was obtained from Sigma Chemical (St. Louis, MO) as the sodium salt. Explants were treated with the standard disinfestation treatment listed using sodium hypochlorite (NaOCl) or NaDCC (0, 100, 300 ppm, or 500 ppm for 24 or 48 hr) as the sources of chlorine. Explants were treated and placed into vials containing NB without rinsing, and scored for visible contamination in 15 days. Treatments with little contamination were repeated but the explants were either coated or not coated with slurry (20 $mL \cdot L^{-1}$ PPM) and placed onto MS medium containing 0 or 5 mls/L PPM.

Results and Discussion

A baseline sterilization procedure was developed that included an initial detergent wash, this step was eliminated in later tests. Predrying of the cut bud explants, an alcohol and acid dip, followed by treatment with a NaOCl solution and organosilica wetting agent were used. The initial use of the sulfuric acid treatment was used to reduce gross contaminant levels. Predrying of the cut stems was done to allow the ends to seal and minimize damage to the vascular tissue. Phloem sieve elements contain a P-protein that polymerizes rapidly on exposure to oxygen (Sjolund et al., 1983). It is believed P-protein functions in wound sealing on the plant. A quick ethanol and acid dip were added to further reduce inoculum levels. This process of using polar and non-polar solutions attempted to dislodge organisms of different cell wall or membrane encapsulating materials. Preliminary experiments comparing hydrochloric and sulfuric acids indicated a similar efficacy (data not shown). The NaOCl soak is a common chlorine treatment used to disinfest plant tissue of contaminants. In preliminary experiments NaOCl was compared with hydrogen peroxide using bud explants from greenhouse-grown trees. The standard disinfestation treatment with NaOCl resulted in 85% contamination, whereas 100% contamination resulted when hydrogen peroxide was used. Peroxide may not be as effective due as chlorine because of a different mode of action, and did not have any antimicrobial action. Silwet L-77, an organosilicone adjuvant with little phytotoxicity to plants, was added to enhance coverage (surface tension) of the biocide solutions.

The degree of contamination was determined by culturing the explants on MS-based medium followed by a subsequent transfer of the explants with no visible contamination after 30 days to nutrient broth. Bacteria and fungi will generally multiply much more rapidly in nutrient broth than MS-based medium. Nutrient broth, both liquid and semi-solid, is used in our laboratory for quality control, and to identify inoculum sources when contamination problems arise.

Slurry Experiments. Initial experiments indicated that field-grown trees were much more difficult to free of culturable contaminants than greenhouse-grown trees. Contaminants on bud explants from greenhouse-grown trees were controlled (>95% clean) with the standard disinfestation procedure followed by culture on medium containing 5 $mL \cdot L^{-1}$ PPM. Dipping into a PPM slurry was not necessary. Phytotoxicity was minimal and buds grew normally. Using buds from field-grown trees, preliminary experiments with PPM (with-

out standard disinfestation treatment) observed that fungi on contaminated explants would grow only on the upper surface of the explant. The hyphae would grow up and over the PPM-containing medium but did not touch the medium. This indicated to us that for effective control the entire explant might have to be coated with PPM-containing slurry. The explants were coated with a slurry of culture medium containing 0.2% agar. In the 0 mL·L^{-1} PPM control 92% of the explants were contaminated (Table 1). Including PPM in the medium provided increasing control to 20 mL·L^{-1}. Phytotoxicity was severe at 20 mL·L^{-1}, was moderate at 10 mL·L^{-1}, and mild at 5 mL·L^{-1}. None of the treatments provided 100% control for field-borne contamination, though the 20 mL·L^{-1} PPM slurry combined with either 5 or 10 mL·L^{-1} PPM medium provided sufficient control for bringing field-derived tissue into culture. An ANOVA with a highly significant 'Medium' main effect (Table 2) confirmed the observation that PPM in the medium was more effective than the slurry.

Liquid incubation experiment. Treatment of bud explants from field-grown trees for five days in suspension culture containing various levels of PPM was not as effective as the PPM-containing slurry and medium treatments (Table 3). Contamination could be reduced by half with a PPM concentration of 20 mL·L^{-1}. Phytoxicity was not a problem with PPM at 5 or 10 mL·L^{-1}, and mild at 20 mL·L^{-1}.

NaDCC experiments. Like the PPM slurry experiments contaminants on bud explants from greenhouse-grown trees were controlled (>95% clean) with the standard disinfestation procedure when NaDCC replaced commercial bleach (i.e., NaOCl) as the chlorine compound. Phytotoxicity was minimal and buds grew normally. Contamination of bud explants from field-grown trees using NaDCC was reduced to as little as 4% (Table 4) compared to 100% for NaOCl. Phytotoxicity was comparable to NaOCl at 300 ppm NaDCC but less at 100 ppm NaDCC. This is consistent with the suggestion by Parkinson et al. (1996) that NaDCC levels < 300 ppm could probably be used, and where as little as 10 ppm were used to sterilize oak somatic embryos. When NaDCC and PPM are combined in treatments the contamination rate is lowered further. If the explants are left in NaDCC from 24 hr and either 300 or 500 ppm NaDCC and 5 ml/L PPM the percent contamination of 10 buds is 0 inbothcases when left for 48 hr the contamination level is 10% for the same concentrations(Table 5). Bud explants were treated with 300 or 500 ppm NaDCC, onto medium containing 0, 2.5 or 5 mL·L^{-1} PPM. The highest phytotoxicity levels of (2.0-2.5) were observed at this level.

Table 1. Contamination levels of citrus bud explants from field grown trees when treated with PPM-containing slurry and culture medium.

Slurry PPM (mls/L)	Medium PPM (mls/L)	Percent of explants with visible contamination[z]
0	0	92
0	5	67
0	10	58
0	20	42
20	0	87
20	5	37
20	10	33
20	20	17

[z]The combined results from two experiments with 12 replications each for a total of 24. Explants showing no detectable contamination at 30 days were placed into nutrient broth and incubated for 14 days at 37°C.

Table 2. ANOVA of % contamination of citrus bud explants from field grown trees treated with PPM-containing slurry and culture medium.

Source	df	SS[z]
Slurry	1	674*
Medium	3	4404**
Slurry X Medium	3	138
Error	8	1092

*, **Significant at P ≤ 0.05 or 0.01, respectively.
[z]Data from averages, transformed by arcsine, calculated from two experiments with 12 replications each.

Table 3. Contamination levels of citrus bud explants from field grown trees treated with PPM for 5 days in suspension culture.

PPM (mls/L)	Percent of explants with visible contamination[z]
0	100
5	96
10	75
20	46

Table 4. Contamination of levels of citrus bud explants from field grown trees treated with NaDCC.

Incubation time (h) in NaDCC	Concentration of NaDCC[z]	Explants with visible contamination[y]
24	0	100
24	100	42
24	300	46
48	0	100
48	100	17
48	300	4

[z]The combined results from two experiments with 12 replications each for a total of 24. Explants showing no detectable contamination at 30 days were placed into nutrient broth and incubated for 14 days.

Table 5. Visual contamination of Valencia bud explants from field grown trees treated with NaDCC and PPM.

	NaDCC	PPM	Phytotoxicity (1-5)	Percent Contamination[x]
	300	0	0-1.0	40
	300	2.5	0-1.0	10
24hr	300	5.0	1.0-2.0	0
	500	0	1.0	30
	500	2.5	1.0-2.0	10
	500	5.0	1.0-2.0	0
	300	0	2.5-3.0	0
48hr	300	2.5	1.0-2.0	10
	300	5.0	2.0	10
	500	0	2.0	10
	500	2.5	2.0-2.5	10
	500	5.0	2.0-2.5	10

[x]The results of 10 explants.

The effectiveness of NaDCC compared to commercial bleach is due to the greater proportion of HOCl. More HOCl is present because the ratio of HOCl to OCl$^-$ ion is dependent solely on the pH of the solution (Fig. 1) (White, 1986). The pH of a 300 ppm NaDCC solution is about 6.8, and 10 for a 20% commercial bleach solution (1% NaOCl). HOCL constitutes 84.54% of the ionic species at pH 6.8, but only 0.34% at pH 10. At pH 10 the primary species is the OCl$^-$ ion. The germicidal efficiency of HOCl is 20 to 300 times more effective, depending on the target organism, than the OCl$^-$ ion (White, 1986), indicating that NaDCC should be more effective than commercial bleach in controlling contaminants. However, in

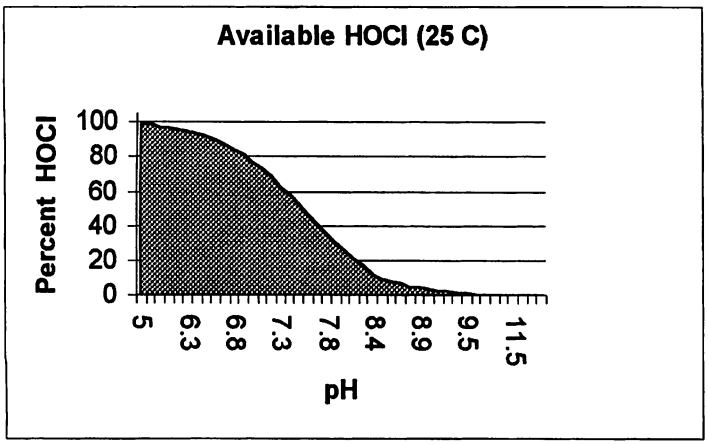

Figure 1. Influence of pH on dissociation of hypochlorous acid to hypochlorite ion at 25 (C. Data calculated from the following: percent HOCl = 100 X [1 + K_i/(H^+)], where K_i is the HOCl dissociation constant of HOCl at 25°C or 2.898 x 10^{-8} moles/liter (White, 1986).

a study by Coates(1985) a comparison of NaDCC and NaOCl found them to be equally effective in control *Pseudomonas aeruginosa*, but that the commercial NaOCl solutions varied greatly in their concentration. Commercial bleach has a problem in that their av.Cl varies greatly with the age of the solution (Coates, 1985). The age of a particular container of bleach is generally unknown. Conversely, because NaDCC is a powder with a shelf life of years, the chlorine content of freshly made solutions is precise and consistent. NaDCC appears to offer some distinct advantages over commercial bleach preparations.

Citrus appears to have mostly external contaminants. The reasons are: 1) in almost all treatments explants that appeared 'clean' on medium almost always tested 'clean' in NB, and 2) contamination was effectively controlled by surface sterilization with NaDCC. Some types of contaminants are more effectively controlled by NaDCC than NaOCl. A combination of NaDCC surface sterilization followed by culture in PPM-containing medium was not any more effective than NaDCC singly. However, including PPM in the medium may provide control in species where explants have internal contaminant. Further work is underway to determine the extent of its efficacy.

Literature Cited

Coates, D. 1985. A comparison of sodium hypochlorite and sodium dichloroisocyanurate products. The J. of Hospital Infection 6 (1), 31-40.

Guri, A. Z. and K. N. Patel. 1998. Compositions and methods to prevent microbial contamination of plant tissue culture media. U.S. Patent 5,750,402.

Leifert, C., C. E. Morris and W. M. Waites. 1994. Ecology of microbial saprophytes and pathogens in tissue culture and field-grown plants: Reasons for contamination problems in vitro. CRC Crit. Rev. Plant Sci. 13:139-183.

Leifert, C. and S. Woodward. 1997. Laboratory contamination management; he requirement for microbiological quality assurance, p. 237-244. In: A. C. Cassells (ed.). Pathogen and microbial contamination management in micropropagation. Kluwer Academic Publishers, The Netherlands.

Marousky, F. J. 1977. Control of bacteria in cut flower vase water. Proc. Fla. State Hort. Soc. 90:294-296.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Niedz, R. P. 1998. Isothiazolone biocides to control microbial and fungal contaminants in plant tissue cultures. HortTechnology (In press).

Parkinson, M., Predergast, M. and Sayegh, A. J. 1996. Sterilization of explants and cultures with sodium dichloroisocyanurate. Plant Growth Regulation 20:61-66.

Pink, D. A. C. and D. G. A. Walkey. 1984. Rapid propagation of Cucurbita pep. L. by culture of meristem tips. Sci. Hort. 24:107-114.

Sjolund, R. D., C. Y. Shih and R. P. C. Johnson. 1983. Freeze fracture analysis of phloem structure in plant tissue cultures. III. P-protein, sieve area pores, and wounding. J. of Ultrastructure Research 82:198-211.

White, G. C. 1986. Handbook of Chlorination, 2nd ed., Van Nostrand Reinhold Company, New York.

Proc. Fla. State Hort. Soc. 111:263-267. 1998.

EVALUATION OF TEBUCONAZOLE FOR CONTROL OF POSTHARVEST DISEASES OF FLORIDA CITRUS

G. E. BROWN AND M. CHAMBERS
Florida Department of Citrus,
Scientific Research Department
700 Experiment Station Road,
Citrus Research and Education Center,
Lake Alfred, FL 33850

Abstract. **Tebuconazole (Elite 45 DF), a fungicide registered preharvest on banana and stone fruits, is being considered for postharvest registration of citrus to control stem-end rot (SER) (*Diplodia natalensis, Phomopsis citri*), green mold (*Penicillium digitatum*), and sour rot (*Geotrichum candidum*). Aqueous suspensions of tebuconazole at 1000-3000 ppm were applied to 'Hamlin' or 'Temple' oranges, 'Sunburst' tangerines, or 'Orlando' tangelos naturally affected by SER and green mold or artificially inoculated with *G. candidum*. Control of SER was obtained with tebuconazole but the efficacy was usually inferior to that of the commercial fungicides thiabendazole or imazalil. However, control of green mold with tebuconazole was comparable to control with these fungicides. Control of sour rot was significantly better with tebuconazole than with sodium orthophenylphenate, the only commercial citrus fungicide with some activity against this disease.**